

Alginate Effects on Human Sperm Parameters during Freezing and Thawing: A Prospective Study

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Abstract

Objective: The main goal was to evaluate the effects of alginate on human sperm parameters during cryopreservation.

Materials and Methods: In this prospective study, twenty-five normozoospermic samples were divided into two groups, encapsulated with 1% alginate and the control group. The samples were then frozen by rapid freezing. Different sperm parameters including motility, normal morphology, viability, acrosome reaction, and DNA integrity, were examined before freezing and after thawing.

Results: All sperm parameters had a significant decrease after thawing compared to before freezing. Our data showed a significant decrease in sperm motility of the alginate group but sperm viability, normal morphology, and DNA fragmentation were similar between the two groups. However, the rates of intact acrosome and native DNA were significantly lower in the control group compared to the alginate group (45.12 ± 11.1 vs. 55.25 ± 10.69 and 52.2 ± 11.92 vs. 68.12 ± 10.15 , respectively, $P < 0.05$).

Conclusion: It seems that alginate can prevent premature acrosome reaction and protect sperm DNA from denaturation during the rapid freezing process.

Keywords: Alginate, Encapsulation, Rapid Freezing, Sperm DNA

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Introduction

Human sperm cryopreservation is a routine practice in assisted reproductive technology (ART) for several reasons including fertility preservation before initiation of radiotherapy, chemotherapy, and vasectomy, for patients with diabetes and autoimmune disorders (1). It could also be used for people with azoospermia without affecting ART outcomes in comparison with fresh controls (2). However, cryopreservation may have detrimental effects on spermatozoa including cold shock, formation of intracellular and extracellular ice crystals, osmotic shock, and production of reactive oxygen species (ROS) that may affect sperm motility (3-5). Spermatozoa are unique cells with a high level of polyunsaturated fatty acid in their plasma membrane, a high number of mitochondria, a low volume of cytoplasm, and antioxidant potential that make the spermatozoa vulnerable to cryopreservation (6).

Encapsulation technology has lately been suggested as a potential favorable method to preserve spermatozoa during cryopreservation. Alginate, an anionic polysaccharide derived from brown seaweed, has been extensively used for several biomedical applications because of its high biocompatibility, relatively low cost, and low toxicity (7). Supplementation with sodium alginate also helped

the metal chelating capacities and free radical scavenging to preserve buffalo sperm during cryopreservation (8). More importantly, cell encapsulation in alginate is an encouraging approach during cryopreservation as cells could be protected against mechanical destruction during ice crystallization (9). It has been shown that alginate mimics the extracellular matrixes for spermatogonial stem cells supporting the potential of their stemness during the cryopreservation (10). Also, the fertilizing potential of cryopreserved sperm using alginate encapsulation did not change in buffalo (11). It was shown that microencapsulation could mechanically inhibit spermatozoa movement. However, it did not negatively impact the intracytoplasmic sperm injection results and a high rate of the vitality of immotile spermatozoa was observed. Also, alginate microencapsulation could preserve spermatozoa from the risk of contamination with foreign material, either spermatozoa or genetic material (12). Controlled-release alginate capsules having boar spermatozoa have also been utilized to increase the time of spermatozoa preservation and maximize the effectiveness of single artificial insemination (13). Also, the alginate microencapsulation method has been used successfully to cryopreserve canine sperm by extending the post-thaw motility, viability, and acrosomal integrity (14).

There are limited data about the cryoprotective effects of alginate on human spermatozoa. Herrler et al. only evaluated the effects of human cell encapsulation on sperm motility and viability (12). They showed that alginate encapsulation using a slow freezing technique decreased human sperm motility while no difference was observed for sperm viability. It seems that using sperm encapsulation in cryopreservation needs to be adjusted. This study aimed to evaluate the effects of alginate encapsulation on human sperm motility, viability, acrosome reaction, and DNA integrity during rapid freezing.

Materials and Methods

Collecting and preparing samples

Twenty-five normozoospermic semen samples were included in this prospective study. Heavy smokers and patients who received antioxidants were excluded from this study. The specimens were obtained from patients who were referred to the Gandhi IVF clinic for infertility workups. The abstinence period was 3-5 days. This study was approved by the Ethics Committee of Tarbiat Modares University (IR.MODARES.REC.1397.198). The direct swim-up method was used to prepare the spermatozoa. The mean age of men was 33.29 ± 3.85 .

Sperm encapsulation into alginate hydrogel

Sperm containing alginate microcapsules were prepared according to the previously described method (12). Briefly, 50 μ L of sperm solution was mixed with 50 μ L of Quinn's Advantage Sperm Freeze (SAGE, USA) for 3 minutes which then was added to 100 μ L sterilized alginate solution (1% w/v, sodium alginate, Sigma-Aldrich, USA). For polymerization, 50-100 μ L aqueous calcium chloride (Sigma-Aldrich, Germany) with a concentration of 102 mmol/L was added dropwise to the as-prepared suspension. After 1 minute, the remaining unreacted crosslinker was removed through washing with 50 μ L of 0.9% w/v sodium chloride. Finally, microcapsules were transferred into the cryotubes containing 100 μ L cryoprotectant medium containing sodium chloride for 3 minutes before cryopreservation.

Scanning electron microscopy and *in vitro* sperm release

Scanning electron microscopy (SEM) was used to evaluate the morphology of the prepared hydrogels without sperm. Samples were first freeze-dried (ALPHA 1-2 LD, UK) overnight before being sputtered with gold for SEM analysis. Evaluation of the *in vitro* rate of sperm releasing was also performed using the previously reported protocol with modification (15). This test was done to estimate the *in vitro* release of spermatozoa after gel encapsulation. For this test, alginate microcapsules were incubated in four-well plates containing 200 μ L of F10 Ham's medium at 37°C (n=3). After incubation for 2, 4, 6, and 24 hours, the entire medium was removed and checked for detection of released spermatozoa. Sperm number was detected by light microscopy with a Neubauer chamber in at least 5

fields. Three replications were assessed for each time point. The percentage of spermatozoa released versus time was calculated using the following formula:

$$\text{Percentage of released sperm} = (\text{number of spermatozoa counted in the medium at a specified time} / \text{total number of encapsulated spermatozoa}) \times 100.$$

Freezing and thawing sperm

In this study, the samples were frozen using the rapid freezing method. As previously described (16), the samples inside cryotubes were first placed horizontally 3 cm above the surface of liquid nitrogen for 30 minutes in the nitrogen vapor and then immersed in the liquid nitrogen. The samples were stored in liquid nitrogen for at least two weeks. During thawing, the cryotubes were first placed in a water bath at 35°C for 2 minutes after removal from the nitrogen tank. In the control group, a pre-warmed culture medium supplemented with human serum albumin was added dropwise and centrifugated. In the alginate group, 150 μ L of 119 mM/L sodium citrate solution (pH=7.5) was added to the cryotube. After 30 seconds, the pre-warmed culture medium supplemented with human serum albumin was added dropwise. After that, the samples were centrifugated. After centrifugation in both groups, the supernatant was removed and the pellet was used for analysis.

Sperm motility, viability, and morphology

Sperm motility was examined according to the WHO guidelines. In each slide, at least 200 spermatozoa were evaluated at six microscopic fields. Each sample was evaluated twice by two different expert technicians and the mean percent was reported for rates of progressive and non-progressive motile and immotile spermatozoa and total sperm motility (progressive+non-progressive). Sperm viability was measured by evaluating sperm membrane integrity using the eosin-nigrosin staining method. The spermatozoa with red/dark pink and white heads were considered live and dead cells, respectively (light microscope 1000x). At least 200 cells were assessed and the rate of sperm viability was reported as percentage. Papanicolaou staining method was used to evaluate the sperm morphology. At least 200 spermatozoa were evaluated reporting morphological abnormalities in the head, midpiece, and tail percentages (light microscope 1000x) (17).

Acrosome reaction

The double staining method was used to evaluate the acrosome reaction. First, samples were fixed in a 1:1 ratio with 3% glutaraldehyde for 30 minutes followed by being centrifuged at 1500 g for 5 minutes. Slides were then stained with 8% Bismarck Brown for 10 minutes, and after rinsing, were being stained with 8% Rose Bengal for 20 minutes. A minimum of 200 spermatozoa was evaluated and the cells with healthy light brown or pink acrosome areas were reported as percentages (18).

DNA denaturation

Acridine orange staining was used to test denatured sperm DNA. First, the sperm sample was placed in Carnoy's fixative solution in a ratio of 3:1 methanol/acetic acid for 2 hours at 4°C and then stained with acridine orange for 10 minutes in the dark. Using a fluorescent microscope, the green spermatozoa were considered healthy (native), and yellow to red spermatozoa were considered abnormal (denatured). A minimum of 200 spermatozoa was examined by a fluorescent microscope (1000x, 460 nm filter) and the rate of spermatozoa with healthy DNA was reported (16).

Sperm chromatin dispersion

Sperm chromatin dispersion (SCD) was used to evaluate the DNA integrity using Sperm DNA Fragmentation Assay [IDEH VARZAN FARDA (IVF Co.), Tehran, Iran]. First, a 20 µL of sperm suspension (15-20 million mL⁻¹) was added to 25 µL of low melting agarose. In the next step, a smear was prepared on a pre-coated glass slide with 65% agarose and kept in the refrigerator at 4°C for 5 minutes. The lysis buffer solution containing hydrogen chloride (HCl) was then poured on the samples in the dark for 7 minutes. After washing the lysis buffer, the slides were immersed in the mercaptoethanol lysing solution, which separates nucleoproteins, for 15 minutes. The slides were then rinsed in distilled water for 5 minutes and the dehydration process was performed with 70, 90, and 100% ethanol. After staining with Wright color, at least 200 spermatozoa were examined for each sample (light microscope 1000x). According to the size of the halo around the head of spermatozoa, the degree of DNA fragmentation was considered. Spermatozoa with no halo or small halo were considered abnormal, while spermatozoa with medium or large halo were considered normal DNA (19).

Statistical analysis

Quantitative data were reported as mean ± standard deviation, median (maximum-minimum). Shapiro-Wilk test was used to evaluate the distribution of data. To analyze the data between the three groups before freezing, alginate, and control, one-way ANOVA with the Tukey test was used. In cases where the distribution was not normal, the Kruskal-Wallis test with the Dunn test was used. The hypothesis was considered one-tailed and the significance level was considered P<0.05.

Results

Scanning electron microscopy

SEM test was performed to evaluate the structure of prepared alginate hydrogels. Figure 1 shows images of alginate hydrogels at two different magnifications. As can be seen, hydrogels have a porous structure with interconnected porosities.

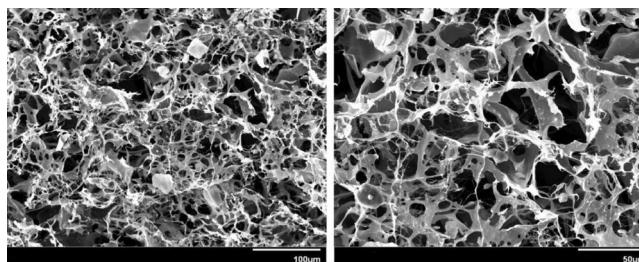


Fig.1: Scanning electron microscopy (SEM) images of alginate hydrogel with different magnifications. Hydrogels have porous structures with interconnected porosities (scale bars: 100 µm and 50 µm in the left and right image, respectively).

In vitro examination of sperm release from alginate capsule

The profile of sperm release percentage from the alginate capsule is shown in Figure 2. Almost 0.01 % of spermatozoa were released after 2 hours. However, the corresponding value reached a constant within 24 hours.

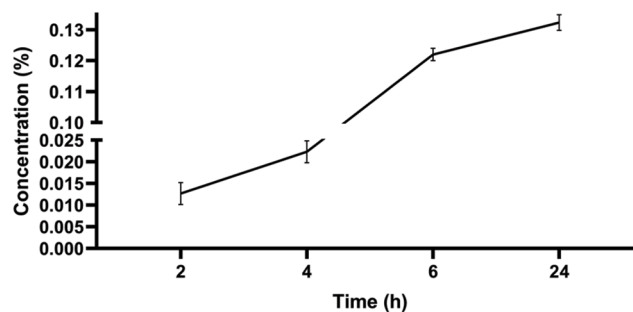


Fig.2: Sperm release profile from alginate microcapsules (n=3). The rate of released spermatozoa had an increasing trend by increasing time and reached a constant value after 24 hours.

Sperm parameters

Semen characteristics included in this study are shown in Table 1. Sperm motility was significantly decreased after thawing compared to before freezing. The rates of progressive motility and total motility were significantly lower in the alginate group compared to the control group. Sperm viability had a significant decrease after thawing but there was the same in the two groups of control and alginate. The percentage of sperm normal morphology after thawing was significantly lower than before freezing. Additionally, after thawing, sperm morphology was preserved in the alginate group compared to the control group, but no significant difference was observed between the two groups. The cryopreservation induced the acrosome reaction. However, the rate of spermatozoa with intact acrosome was significantly higher in the alginate group compared to the control group. The rate of native DNA in spermatozoa after thawing was significantly lower

than before freezing. This reduction was improved in the alginate group and a significant difference was observed between the alginate and control groups. The rate of DNA fragmentation significantly decreased after thawing. This

reduction improved in the alginate group but there was no significant difference between the alginate group and the control group (Table 2). Figure 3 shows acridine orange, acrosome reaction, and SCD tests.

Table 1: Semen characteristics included in this study

Parameters	Mean	Minimum	Maximum	SD
Male age (Y)	33.29	23	39	3.85
Count (10 ⁶ /mL)	140.25	90	182	26.15
Progressive motility (%)	61.45	43	70	6.9
Non-progressive motility (%)	16.25	6	30	6.27
Total motility (%)	77.7	54	89	8.37
Immotile (%)	22.29	11	46	8.37
Normal morphology (%)	7.8	4	15	1.32
Round cell (10 ³ /mL)	427.16	200	950	203.31

SD; Standard deviation.

Table 2: Comparison of the mean percentage of sperm parameters (± standard deviation) between different experimental groups

Parameters	After swim-up	Control group	Alginate group
Progressive motility	83.87 ± 3.8 ^{ab}	47.79 ± 16.28 ^{bc}	13.75 ± 10.64 ^{ac}
	84.5 (76-90)	55 (13-69)	10 (0-40)
Total motility	92.83 ± 3.62 ^{ab}	65.33 ± 15.67 ^{bc}	29.91 ± 15.01 ^{ac}
	93 (85-98)	71 (26-91)	28.5 (7-65)
Viability	97.33 ± 1.97 ^{ab}	89.83 ± 4.93 ^b	89.58 ± 3.33 ^a
	97 (91-99)	90 (75-96)	89 (85-97)
Normal morphology	17.17 ± 4.18 ^{ab}	8.83 ± 3.54 ^b	11.13 ± 3.2 ^a
	16.5 (10-32)	9 (4-19)	10 (6-18)
Intact acrosome	70.16 ± 7.11 ^{ab}	45.12 ± 11.1 ^{bc}	55.25 ± 10.69 ^{ac}
	70 (54-84)	42 (29-66)	52 (37-75)
Native DNA (AO)	77 ± 9.47 ^{ab}	52.2 ± 11.92 ^{bc}	68.12 ± 10.15 ^{ac}
	76.5 (58-94)	51 (33-80)	67.5 (49-84)
Normal DNA (SCD)	90.2 ± 6.07 ^{ab}	79.45 ± 9.26 ^b	81.95 ± 7.88 ^a
	92 (68-96)	81 (60-92)	85.5 (65-93)

Data are presented as mean ± SD, median (min-max). Similar letters have significant difference. AO; Acridine orange and SCD; Sperm chromatin dispersion test.

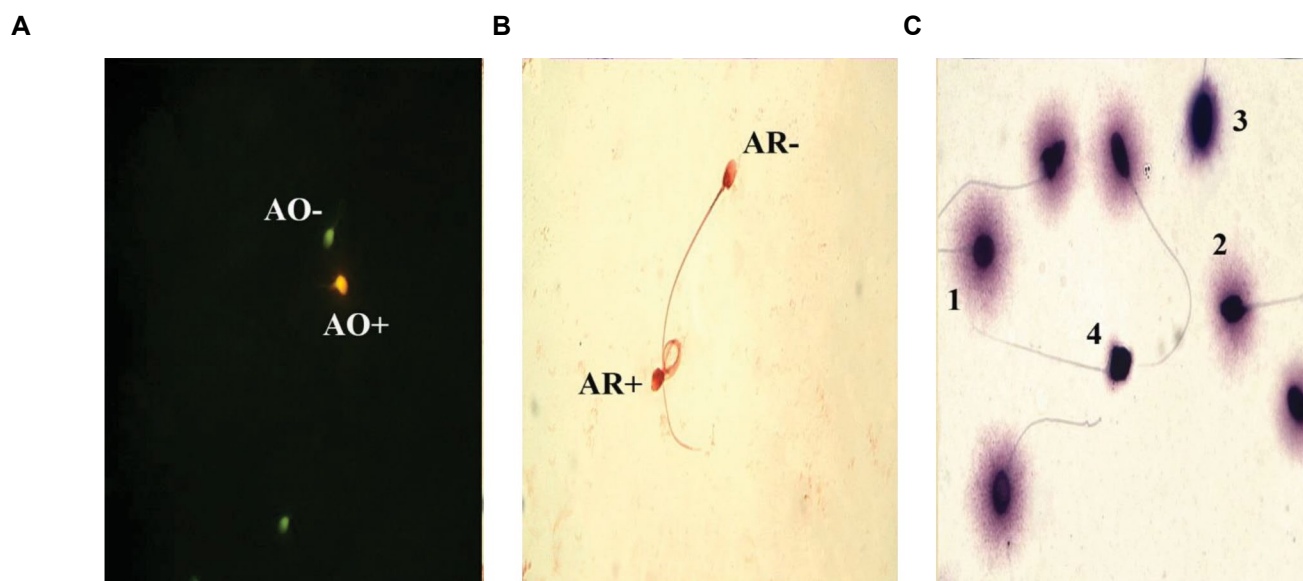


Fig.3: Acridine orange, acrosome reaction and sperm chromatin dispersion test. **A.** Acridine orange shows the orange sperm with DNA denaturation (AO+) and green native DNA (AO-), **B.** Double staining shows the sperm with brown head (acrosome intact, AR-) and dark brown and conical head (acrosome reacted, AR+), **C.** Sperm chromatin dispersion test shows different patterns of halos; 1; A normal DNA with a large halo, 2; A normal DNA with a medium halo, 3; Abnormal DNA with small halo, and 4; Abnormal DNA without halo (1000 \times).

Discussion

Cell encapsulation with alginate is a promising method of preventing unwanted effects during cryopreservation. Our results showed that, after thawing, the rate of sperm progressive motility was significantly lower in the alginate group compared to that of the control group which was in line with a previous study that used 7 mg/dL alginate to encapsulate human sperm *in vitro* under slow freezing (12). It seems that one of the factors that decrease sperm motility may be the presence of alginate particles on the surface of sperm. The cytoskeletal structure of the sperm flagella is composed of fibrous sheaths which play an active role in sperm motility (20, 21). When the alginate residue is retained on the sperm, it may impair the active movement of the sperm tail and reduces motility. Torre et al. encapsulated porcine semen with 0.5% alginate and reported a decrease in sperm motility due to the remaining residual alginate particles on the sperm which impaired sperm motility (22). In fact, alginate hydrogel may be affected by many factors such as crosslink concentration, type of alginate, alginate concentration, cell encapsulation method, and protocol of hydrogel degradation. Each of these factors can affect sperm motility alone. Moreover, most damages occur rapidly after the thawing stage (23). During thawing, cryoprotectants inside the cell cannot leave the cell quickly, but water enters the cell faster leading to osmotic shock causing cell swelling and sperm damage. Alginate, which acts as a matrix around the spermatozoa and protects the sperm membrane, is removed during thawing.

According to the results of the present study, the sperm viability was the same between the alginate and control groups. As mentioned earlier, alginate is a biodegradable polymer with high biocompatibility, with a prominent

potential to form a three-dimensional matrix around the cell similar to the extracellular matrix. This type of porous matrix can maintain the desired level of cell viability in any *in vitro* and *in vivo* environment (24). Alginate creates a unique structure that facilitates the transport of signaling molecules and nutrients. It was shown that poly (propylene fumarate)-co-alginate resists the penetration of ROS (25). However, according to Pirnia et al., spermatogonia stem cell viability in the alginate-containing group was significantly reduced compared to the control group. According to this study, the probable cause of the decrease in viability is in the thawing step after removing alginate hydrogel and exposure to ROS (10). In addition, Kumar et al. (8), supplemented semen extender of buffalo with alginate and cryopreserved the samples with a programmable biological freezer. They showed that alginate maintains membrane integrity and increases sperm viability.

Regarding sperm morphology, the rate of normal sperm morphology in the alginate group was similar to the control group. Osmotic stress may affect sperm morphology. Permeable cryoprotectant agents must penetrate the cell to play protective roles and this addition before freezing and removal in the thawing step causes severe osmotic volume changes, which in turn may cause cell injury (26). These rapid changes in cell osmolality have caused the shape and structure of the sperm membrane to change especially in the form of deformities in the sperm head and the twisting of the sperm tail. According to Torre et al. (15) the percentage of porcine sperm morphological abnormalities in both encapsulated and control groups was not significantly different concluding that the presence of sperm morphological abnormalities does not depend on the storage process in the alginate.

Based on our results, the acrosome reaction rate after the freeze-thawing process was significantly decreased in the alginate group compared to that of the control group. During the cryopreservation, due to temperature changes and increased ROS, the structure of polyunsaturated fatty acids and cholesterol is disrupted, which in turn increases the membrane's permeability to calcium and ROS, subsequently activating phospholipase A2, leading to the onset of an acrosome reaction (27). It was shown that adding sodium alginate to the cryopreservation medium prevents sperm membrane changes and maintains membrane integrity, which in turn preserves the acrosome membrane and prevents premature acrosome reaction (8).

Our data showed that acridine orange and SCD results were not in line with each other in the alginate group. In the group containing alginate, the rate of DNA denaturation and DNA fragmentation decreased compared to the control group, which significantly differed in DNA denaturation. It was shown that there was a lack of relationship between SCD and acridine orange (28). The cut-off value of acridine orange (native DNA) is greater than 50% (29) and all groups in our study had native DNA greater than the cut-off value. It suggested that acridine orange is reliable when there is a high level of DNA damage (28). Two important factors of DNA fragmentation and DNA damage are oxidative stress and apoptosis (30). In fact, the increase in ROS production during cryopreservation is the cause of oxidative stress leading to DNA fragmentation. In the thawing step, various factors such as temperature, osmotic stress, and the use of centrifuges cause defects in the sperm mitochondrial membrane and ROS production (31). Removing alginate at the thawing step makes the spermatozoa more prone to oxidative stress compared with the control group. According to Pirnia et al. (10), the use of 1% alginate hydrogel for encapsulation of spermatogonia stem cells can regulate the differentiation of these cells and maintain their pluripotency of them during slow freezing by regulating *Lin28a* and *Sall14* genes. The potential role of encapsulation is to maintain sperm viability while allowing their gradual release (32). Our data showed that *in vitro* sperm release was very low and the corresponding value reached constant within 24 hours confirming the stability of the hydrogel structure in F10 Ham's medium over time (15). Microencapsulation of semen has been suggested as a method of choice for a gradual release of spermatozoa within the female reproductive or extended storage (33). The latter was regarded in this study. Here, we did not observe the fast release and the total *in vitro* sperm release was very low. We do not expect many releases after effective crosslinking of the hydrogel as the calcium chloride is making links between the alginate branches. This observation confirms the great integrity of the prepared hydrogels preventing the burst effect of the sperm release. We also achieved gradual release which further approves the stability of the hydrogel structure. More importantly, different parameters including concentrations of calcium ions for making the capsules could affect gel strength and thickness of the alginate membrane which in turn impacts the spermatozoa release

behavior (22). Further studies are required to evaluate the effect of sperm species, alginate type, and the cross-linkers as well as their concentration for optimal sperm release. In this study, we evaluated the impact of alginate encapsulation on normozoospermic samples. It should be noted that pre-cryo sperm parameters including motility and viability are useful to predict post-thaw outcomes (34). It is suggested to evaluate the probable effects of alginate encapsulation on abnormal sperm samples in the next research.

Conclusion

According to the results of this study, alginate can prevent sperm premature acrosome reaction and protect sperm DNA from denaturation during the rapid freezing process. More studies should be performed for optimization of alginate for application in sperm cryopreservation.

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Authors' Contributions

I.H., N.B.; Designed the study and revised the manuscript. S.F.; Conducted experiments and wrote the manuscript. I.H.; Conducted statistics. All authors read and approved the final manuscript.

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